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Gene Expression Analysis of Interleukin-8, Interleukin-10, and Interleukin-18 among Chronic Otitis Media Patients with Effusion

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Abstract: Immune responses have been linked to middle ear effusion in patients with chronic otitis media with effusion (COME). Despite the fact that different cytokines play a role in immunologic responses in OME patients. Through the period extending from August 2022 until April 2023, under sterilized conditions 110 clinical specimens (ear discharge) collected by Otorhinolaryngologists from outpatients and inpatients with chronic otitis media (COM) with effusion whose visited Ear-Nose-Throat (ENT) consulting in Ghazi Al-Hariri hospital for specialized surgeries in medical city. The specimens collected from adults (15-55 years) and children (5-9 years). According to the results of the biochemical tests and molecular identification, out of the 110 samples of ear exudates examined, 49 (44.5%) specimens contained bacterial pathogens including *Staphylococcus aureus* (no=6, 5.5%), and *Pseudomonas aeruginosa* (no=17, 15.5%),16 *Moraxella catarrhalis* (14.5%) and 6 *Streptococcus pneumoniae* (5.5%) and 4 *Haemophilus influenzae* (3.6%). Using the delta delta ct method, the gene expression of three interleukins (IL-8, IL-10, and IL-22) was assessed in patients with three species (*S. pneumoniae*, *M. catarrhalis*, and *P. aeruginosa*) and contrasted the outcomes with those of a healthy control group. The gene expression findings of three Interleukins among Chronic Otitis media patients infected with *P. aeruginosa*, where the maximum level in effusion was 5.6 for IL-8, follow by 4.9 for IL-22, while the lower levels were for IL-10 (2.1) in blood and (3.2) in effusion, while with *M. catarrhalis*, the findings demonstrated that the expression of the three interleukins in blood and effusion exhibited significant raising in comparison with the healthy control, where the maximum level in effusion was 5.5 for IL-8, follow by 5.3 for IL-22, while the lower levels were for IL-10 (1.8) in blood and (2.2) in effusion. Also, the results of *S. pneumoniae* revealed that there was an increasing of the fold change of gene expression of interleukin-22 in blood (5.2) and effusion (6.1) in comparison with other interleukins and the healthy control, and the maximum level in effusion was 6.1 for IL-22, follow by 3.6 for IL-8. In conclusion, the results indicated to the importance of IL-22 in all bacterial infections and especially with *S. pneumoniae* and IL-8 for *P. aeruginosa*, *M. catarrhalis*. Also, the lower levels of expression of mRNA for IL-10 were related to all bacterial infections.

Key words: Chronic Otitis media, Effusion, Gene expression, Interleukins.

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Introduction

Otitis media is a general term for a group of inflammatory disorders with varying clinical manifestations that affect the mucosal lining of the middle ear. The most frequent cause of it is fluid accumulation behind the eardrum brought on by an obstruction in the Eustachian tube. Because children's Eustachian tubes are shorter, more

horizontal, and composed of more flaccid cartilage than those of adults, which can impede their opening, otitis media is more common in children (1). In both high- and low-income nations, otitis media is the most common reason for medical attention seeking and antibiotic prescriptions (2). By the time they are two years old, about 70% of babies have had at least one episode of otitis media, and 20–30% have recurrent acute otitis media (RAOM). Since RAOM cause pain and discomfort in children, it has significant impact on their families and places a significant financial burden on society. As such, it is an issue that is highly relevant to clinical practice (3).

Usually multiple otopathogen colonization in the nasopharynx and middle ear. An environment that facilitates bacterial attachment and colonization, adhesion to cells, and middle ear invasion is produced by viral infections of the nasopharynx (4). The primary cause of both AOM and RAOM was thought to be bacterial otopathogens, specifically *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Haemophilus influenzae*, and *Moraxella catarrhalis* (5). While *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Klebsiella* spp., and *Escherichia coli* are other species commonly isolated from the middle ear fluid of children with Chronic suppurative otitis media (CSOM) as experiencing this condition (6), while, the most common fungal detection which causing of CSOM was *Aspergillus* spp. and *Candida* spp (7). Several local studies indicated to the role of *P. aeruginosa* as main pathogen in otitis media with high resistance to antibiotics and strong biofilm formation (8, 9).

A key factor in the prompt resolution of OM is innate immune responses. On the other hand, persistent inflammation, chronic OM, and inadequate bacterial clearance can result from dysregulation in the innate immune system network. The absence of key players in the aforementioned pathways, including ILs, TLR, NLR, MyD88, and TNFα, increases the risk of chronic OM. Therefore, these pathways play a key role in both the pathophysiology of chronic OM and the typical recovery from OM (10).

The functions of interleukin (IL)-8, -10, and -22 in Chronic Otitis Media with Effusion (COME) are poorly understood. As a result, this study assessed the three cytokines' expression levels in COME patients as well as their correlation with the presence or absence of specific pathogenic bacteria.

Materials and Methods

Subjects study design

A total of 110 patients diagnosed with chronic otitis media with effusion (COME) between March 2023 and June 2023 at Medical City Hospital for tympanomastoidectomy and/or ventilation tube insertion contributed samples. When undergoing otoscopic examination, OME was recognized by the presence of an amber-colored tympanic membrane, and when performing impedance audiometry, the presence of a B- or C-type tympanogram.

Ethical Considerations

All patients, along with their parents or guardians, were informed about the study's purpose and were asked to provide written informed consent for the use of their samples. The regional institutional review board gave its approval to the research protocol.

Samples

Before surgery, patients with exudative OM had a tympanostomy

tube inserted, a radial incision made in the anterior inferior quadrant of the tympanic membrane, and the external auditory canal cleaned with potadine solution. Middle ear effusion fluid (MEEF) was aseptically aspirated while stopping the bleeding using a collector. Granulation tissue was removed from patients with COM and cholesteatoma from patients with CholeOM during tympanomastoidectomy. The samples were stored at -80 °C after being transferred to Eppendorf tubes.

RNA extraction and real-time PCR procedures

TRIzol reagent was used to extract total RNA from patient samples in accordance with the manufacturer's instructions (Promega, USA). Skin sample RNA was isolated with Qiagen (Germany) RNeasy Mini kits. Following the manufacturer's instructions, 1µg of total RNA was converted to first-strand cDNA using a reverse transcription system with random hexamers (Promega, Madison, WI, USA). A StepOnePlus realtime PCR system was used to conduct real-time PCR. 1µl of cDNA, 10µl of Power SYBR Green PCR Master Mix (Applied Biosystems, USA), 2µl of particular primers (Table 1), and 7µl of PCR-grade water were added to each 20µl reaction mixture. The amplification protocol included 40 cycles of denaturation at 95 °C for 15 s, annealing and extension at 60 °C for 1 min, and an initial denaturation at 95 °C for 10 min. The crossing points of the target genes with β-actin were calculated using the formula 2–(target gene–β-actin), and relative amounts were quantified.

Table (1): Oigonucleotide sequences of primers used in this study.

Name of gene	Sequences	Annealing temperature (C^O)	Product size (pb)	Reference
IL-22	F:5'-GCAGGCTTGACAAGTCCAACT-3'	60	70	(11)
	R:5'-GCCTCCTTAGCCAGCATGAA-3'			
IL-10	F:51-GCCTAACATGCTTCGAGATC-31	60	105	(12)
	R:51-TGATGTCTGGGTCTTGGTTC-31			
IL-8	F:5-AAG AGA GCT CTG TCT GGA CC-3'	55	300	(13)
	R:5-GAT ATT CTC TTG GCC CTT GG-3'			
β-actin	F:5'-GCGAGAAGATGACCCAGATC-3'	60	77	(11)
	R:5'-GGATAGCACAGCCTGGATAG-3'			

Bacterial Identification

Sterile cotton swabs were dipped in Stuart transport medium to collect of discharge samples from patients with COM, as well as effusion fluid samples from patients with OME. These samples were inoculated into liquid thioglycollate medium (Himedia, India) and solid blood agar medium. After that, the cultures were left to incubate for twenty-four hours at 35°C. Gram staining and biochemical testing were used to identify the bacteria that formed colonies.

Statistical analysis

All of the data were analyzed using SPSS 20.0, a statistical software. Following the use of the Kruskal-Wallis test or the Mann-Whitney U-test to determine group differences, post hoc comparisons were carried out. Statistical significance was established at $p<0.05$.

Results and Discussion

By using the delta delta ct method, the gene expression of three interleukins—IL-8, IL-10, and IL-22—was assessed in patients with chronic otitis media infection (*S. pneumoniae*, *M. catarrhalis*, and *P. aeruginosa*) and

contrasted the outcomes with those of a healthy control group. Interleukins' mRNA expression levels were found in the infected patients' effusion and peripheral blood mononuclear cells (PBMCs). Figures 1 to 3 and table 2 summarized the findings of the gene expression as a fold change of interleukins.

Table (2): Fold change of *IL-8, IL-10* and *1L-22* gene expression with difference types of bacterial infection.

Type of bacteria	Type of sample	IL-8	IL-10	IL-22
Control		1.0 ±0.00 c	1.0 ±0.00 d	1.0 ±0.00 e
Pseudomonas aeruginosa	Blood	3.6 ±0.37 b	2.1 ±0.16 bc	3.9 ±0.34 cd
	Effusion	5.6 ±0.54 a	3.2 ±0.27 a	4.9 ±0.38 bc
Moraxella catarrhalis	Blood	4.1 ±0.39 b	1.8 ±0.14 cd	3.6 ±0.27 d
	Effusion	5.5 ±0.48 a	2.2 ±0.19 bc	5.3 ±0.51 ab
Streptococcus pneumoniae	Blood	3.2 ±0.27 b	2.4 ±0.23 abc	5.2 ±0.47 ab
	Effusion	3.6 ±0.41 b	2.8 ±0.29 ab	6.1 ±0.59 a
L.S.D. (P-value)		1.056 ** (0.0043)	0.966 ** (0.0081)	1.147 ** (0.0036)
** Means having with the different letters in same column differed significantly. (P≤0.01).				

The results of gene expression of three Interleukins among Chronic Otitis media patients infected with *Pseudomonas aeruginosa* (Figure1). The findings revealed that there was an obvious increasing of the three interleukins in blood and effusion in comparison with the healthy control (1.1 fold), where the increasing ranges was from 2.1 to 5.6 as fold change. Also, it was found that the levels of expression of mRNA in effusion (3.2-5.6) were more than those of the blood samples (2.1-3.9), and the maximum level in effusion was 5.6 for IL-8, follow by 4.9 for IL-22, while the lower levels were for IL-10 (2.1) in blood and (3.2) in effusion (Figure1).

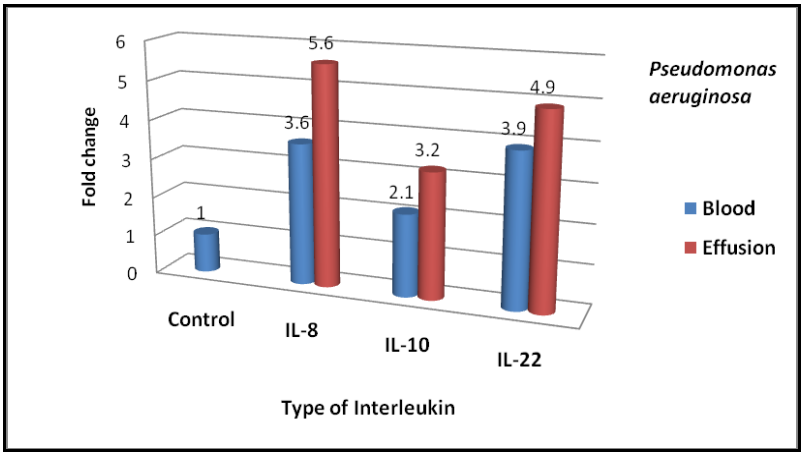

Figure (1). The mRNA expression levels (fold change) of Interleukins (IL-8, IL-10 and IL-22) in Blood and Effusion of Chronic Otitis media patients infected with *Pseudomonas aeruginosa* compared to the control group. (The results represent the mean of Five patients).

The secondary cytokine L-8 reduces middle ear inflammation by acting as a chemotaxic agent on neutrophils. Furthermore, during the acute inflammatory response, IL-stimulates the synthesis of adhesion molecules required for neutrophil attachment and migration. IL-8 can damage tissue and release lysosomal enzymes, which can lead to chronic ear inflammation (14).

The results of gene expression of three Interleukins among Chronic Otitis media patients infected with *Moraxella catarrhalis* (Figure 2), demonstrated that the expression of the three interleukins in blood and effusion exhibited significant raising in comparison with the healthy control (1.1 fold), where the increasing range was from 1.8 to 5.5 as fold change. Also, it was found that the levels of expression of mRNA in effusion (2.2-5.5) were more than those of the blood samples (1.8-4.1), and the maximum level in effusion was 5.5 for IL-8, follow by 5.3 for IL-22, while the lower levels were for IL-10 (1.8) in blood and (2.2) in effusion (Figure 2).

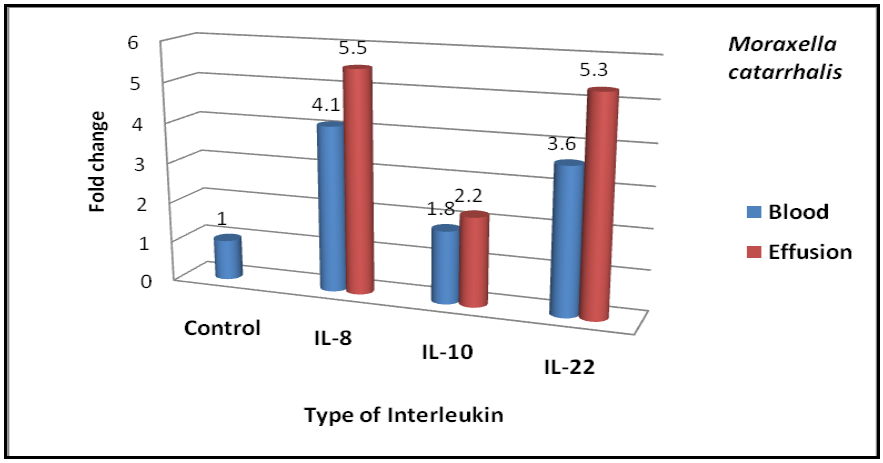

Figure (2). The mRNA expression levels (fold change) of Interleukins (IL-8, IL-10 and IL-22) in Blood and Effusion of Chronic Otitis media patients infected with *Moraxella catarrhalis* compared to the control group. (The results represent the mean of Five patients).

The results of gene expression of three Interleukins among Chronic Otitis media patients infected with *Streptococcus pneumoniae* (Figure 3).

It was found that there was an increasing of the fold change of gene expression of interleukin-22 in blood (5.2) and effusion (6.1) in comparison with other interleukins and the healthy control (1.1 fold), where the increasing range of other interleukins was from 2.4 to 3.6 as fold change. Also, it was found that the levels of expression of mRNA in effusion (2.4-5.2) were more than those of the blood samples (2.8-6.1), and the maximum level in effusion was 6.1 for IL-22, follow by 3.6 for IL-8, while the lower levels were for IL-10 (2.4 in blood and 2.8 in effusion) (Figure 3).

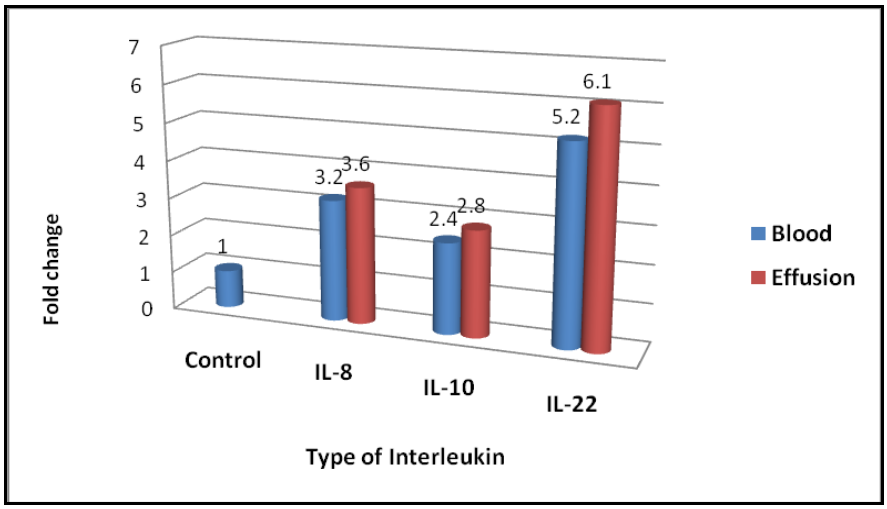

Figure (3). The mRNA expression levels (fold change) of Interleukins (IL-8, IL-10 and IL-22) in Blood and Effusion of Chronic Otitis media patients infected with *Streptococcus pneumoniae* compared to the control group. (The results represent the mean of Five patients).

The two most frequent bacterial pathogens linked to CSOM, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, form biofilms with other otopathogens and increase innate inflammatory responses, even in the face of suitable intervention, these factors might be to blame for the chronic nature of OM and its progression to CSOM. Increased inflammation is indicated by high levels of IL-8 in the middle ear fluid and higher levels of TNF, IL-1β, IL-6, and interferon-γ (IFNγ) in the middle ear mucosa when compared to patients with chronic OME (15).

The present study indicated to the important role of the inflammatory interleukins (IL-8, IL-10, and IL-22) among patients Chronic Otitis Media infection (*P. aeruginosa*, *M. catarrhalis*, and *S. pneumoniae*), and the expression of these cytokines in effusion was in high levels which demonstrated the involvement of these interleukins in the chronic infection of otitis media. The results revealed to the important of IL-22 in all bacterial infections and especially with *Streptococcus pneumoniae* and IL-8 for *P. aeruginosa*, *M. catarrhalis*. Also, the lower levels of expression of mRNA for IL-10 were related to all bacterial infections. (11) examined the mRNAs for three groups of patients with OM in relation to IL-17, IL-22, IL-23, and IL-26. They discovered that compared to patients with cholesteatomatous OM and chronic OM, all of these mRNAs were significantly higher in patients with otitis media with effusion. The elevated levels of all four mRNAs in OME patients were unaffected by the type of hearing loss or by the presence or absence of bacteria. Inducing distinct innate immune responses in epithelial cells, IL-22 is essential for the host's defense against various invasive pathogens, including *Citrobacter rodentium* and *Klebsiella pneumonia*. IL-22 also preserves mucosal homeostasis and safeguards tissue integrity. Conversely, the proinflammatory cytokine IL-22 can exacerbate inflammatory responses and result in tissue damage, as demonstrated by the IL-22-dependent necrosis of the small intestine during infection with *Toxoplasma gondii* **(**16).

In *P. aeruginosa*-infected mice, the level of IL-22 was momentarily elevated, and further IL-22

administration lessened the mice's lung damage by reducing local neutrophil infiltration. Additionally, IL-22 promotes the production of IFN-λ in the lungs, which inhibits the release of inflammatory mediators like IL-1β. Remarkably, IL-22 can be broken down by serine protease-3 released by neutrophils as well as protease IV released by *P. aeruginosa*. As a result, they aid *P. aeruginosa*'s immune escape and may cause pulmonary bacterial colonization as well as ongoing declines in respiratory function (17). There were 70 pediatric patients in the study cohort (46 boys and 24 girls). During surgery, samples of the effusion fluid were taken, and real-time PCR was used to measure the amounts of IL-17 and IL-22 mRNA. Patients with sinusitis had higher levels of IL-17 and IL-22 mRNA than those without, but only IL-22 mRNA levels were significantly different. Compared to serous fluid samples, mucoid and purulent middle ear fluid samples had a significantly higher level of IL-22 expression (18).

The authors proposed the following theory regarding chronic otitis media with effusion (COME): 1. the leukocyte build-up in the middle ear cleft and 2. leukocyte activation in situ followed by tissue damage. Furthermore, the authors postulated that interleukin-1 (IL-1) and tumor necrosis factor (TNF) regulate IL-8 expression, at least partially (19). Therefore, it is abundantly evident that IL-8 is continuously found in the MEE of kids with COME and that it has a strong correlation with IL-1 beta and TNF-alpha levels, two substances that are known to induce the production of IL-8. The authors' hypothesis regarding the intimate involvement of IL-1 beta, TNF-alpha, and IL-8 in the inflammatory cascade in the middle ear is supported by these results, which also point to the potential for future therapeutic intervention in the treatment of otitis media with effusion (OME) through regulation of these cytokines (20).

AOM has been linked in a prior study to increased MEE levels of IL-6 and IL-8, indicating a potential role for these cytokines in the pathophysiology of AOM. High specificity could be used to distinguish bacterial from nonbacterial cases based on the elevated levels of both cytokines. Additionally, elevated MEE levels of IL-6 are specific for *S. pneumoniae* infections, whereas elevated levels of IL-8 are sensitive for *H. influenzae* infections. Therefore, measuring MEE levels of both cytokines could facilitate early therapeutic regimen initiation and speed up the diagnostic process (21).

Serum levels of interleukin 1α (IL-1α), interleukin 6 (IL-6), and interleukin 8 (IL-8) were assessed before surgery and compared between patients with chronic suppurative otitis media (CSOM), cholesteatoma, and recurrent cholesteatoma. Interleukins were found in higher concentrations in the serum of all the groups compared to the healthy control group. The highest values of IL-6 and IL-8 were found in patients with CSOM, whereas the highest values of IL-1α were found in patients with cholesteatoma recidivism. Thus, we can deduce that inflammatory mediators play a significant role in the pathophysiology of cholesteatoma and CSOM by maintaining a systemic and local inflammatory response (22).

The results of (23) showed that TGF-beta1 and IL-10, two significant immunoregulatory mediators, are involved in the middle ear inflammatory response, particularly in the long-term course of adult OME. All MEEs and plasmas from patients with chronic OME had higher concentrations of IL-10 ($P < 0.05$) and TGF-beta1 ($P < 0.01$)

than those from patients with acute OME. Both in acute and chronic OME ($P < 0.01$), compared to plasmas, MEEs had a noticeably higher IL-10 concentration. The presence of bacteria detected by either culture or PCR was strongly correlated with the levels of interleukin (IL)-1α, IL-17, IL-1β, fibroblast growth factor basic, and tumor necrosis factor in middle ear fluid, whereas IL-1RA, IL-3, IL-6, IL-8, CCL3, CCL4, and granulocyte-colony stimulating factor correlated significantly with real-time PCR values. Viral nucleic acid levels were substantially correlated with CXCL10, CXCL9, CCL2, and TRAIL. In conclusion, the continued presence of bacterial and viral pathogens may contribute to the ongoing inflammation in SOM. While viruses primarily produced the interferon-induced chemokines CXCL9 and CXCL10, bacteria induced a widespread inflammatory response (24).

The results of the gene expression of IL-8 and IL-22 in effusion of patients with chronic otitis media from adults and children (5-9 years) were summarized in table 3 and Figure (4), demonstrated that there was a high level of mRNA of IL-8 among children effusion in comparison with adults, while the level of mRNA of IL-22 among children effusion and adults exhibited non- significant differences.

Table (3): Fold change of *IL-22* and *IL-8* gene expression according to the age groups.

Age Groups	IL-22	IL-8
Control	1.00 ±0.00 b	1.00 ±0.00 c
Patients/ Adults	4.9 ±0.35 a	5.3 ±0.47 b
Patients/ Children	5.6 ±0.58 a	8.8 ±0.71 a
L.S.D. (P-value)	0.959 ** (0.0006)	1.372 ** (0.0001)
Means having with the different letters in same column differed significantly. ** ($P \leq 0.01$).		

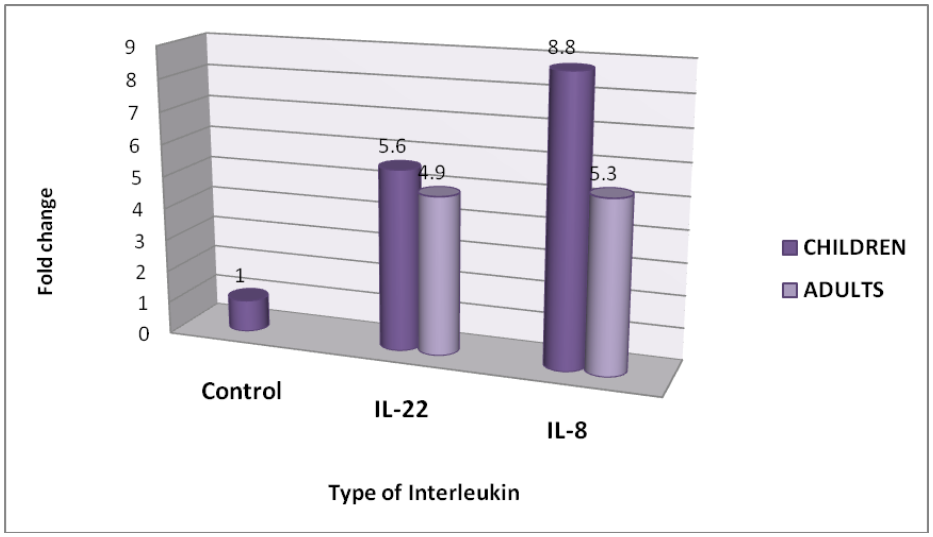

Figure (4). The mRNA expression levels (fold change) of Interleukins (IL-8, and IL-22) in Effusion of Chronic Otitis media patients infected with *P. aeruginosa* compared to the control group. (The results represent the mean of five patients).

The novel immortalized pediatric MEE lines are superior to the currently available adult MEE line in terms of baseline expression and response of

OM-relevant cytokines, indicating that they are better models for pediatric OM. These variations are in line with phenotypes seen in the corresponding primary cultures as well as variations in innate immunity between adults and children, which are assumed to put kids at higher risk of having an exaggerated inflammatory response. These cell lines hold potential for future basic and translational research and serve as helpful new in vitro models for the study of OM (25). Many local studies revealed that the bacterial infections of otitis media exhibited the need to new generations of antibiotics because the high resistance among used antibiotics (26, 27).

Conclusion

The pathophysiology of OM involves high levels of mRNA for IL-8, IL-10, and IL-22. Although IL-8 and IL-22 played a significant role in the chronic condition of adult patients with otitis media, the levels of all three mRNAs are significantly higher in COME patients than in healthy individuals.

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